Inheritance studies implicate a genetic mechanism for apparent sex-reversal in Chinook salmon

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Abstract- The apparent increase in altered sexual differentiation in Pacific salmon is a growing concern. Previous studies suggest that incongruence between genetic and phenotypic sex in Chinook salmon (*Oncorhynchus tshawytscha*) may be due to altered sexual differentiation through exposure to endocrine disrupting chemicals (EDCs). Artificial crosses between genotypically normal Chinook salmon, or between genotypically normal males and apparent sex reversed males (XY-females) were performed to test the validity of OtY1 and growth hormone pseudogene (GH-Ψ) genetic markers as indicators of phenotypic sex in fall-run Chinook salmon. The offspring produced were genotyped with the Y-chromosome specific markers, and were dissected to observe gonad morphology. The results of the breeding experiments indicate that approximately half of the phenotypic female offspring of XY-females have a male

genotype according to both Y-chromosome markers. These results refute an earlier hypothesis that phenotypic female Chinook salmon with a male genotype (XY-females) are the result of altered sexual differentiation due to EDC exposure. Instead, either the OtY1 and GH-Ψ markers have recombined between the Y- and X-chromosomes or an autosome, or a mutation has inactivated the sex-determining region of the Y-chromosome. In none of the 2384 fish evaluated did the genetic markers contradict one another in a single individual. These results present evidence that both OtY1 and the GH–Ψ genetic markers appear to not be diagnostic for sex in fall-run Chinook salmon in the Central Valley of California.

Introduction

Recent data for Chinook salmon (*Oncorhynchus tshawytscha*) in the Pacific Northwest (Nagler et al. 2001; Chowen and Nagler 2004) and in California (Williamson and May 2002) suggest that observed incongruence between genetic and phenotypic sex may be due to altered sexual differentiation. The potential for this phenomenon to adversely impact salmon population persistence is a growing concern. Phenotypic female Chinook salmon that have a male genotype are fertile and cannot be visually distinguished from genetically normal females (Williamson and May 2002). These fish may be inadvertently incorporated into artificial propagation programs of hatcheries residing in watersheds that have populations with a high frequency of sex-reversal. This could exacerbate population level genetic effects posed by sex-reversed fish (Williamson and May 2002). Mating between a sex-reversed male (XY-female) and a genetically normal male (Figure 1C) would result in a 3:1, male to female, genotypic and phenotypic sex-ratio in the offspring. One third of the male offspring produced from this cross would be YY. Subsequent reproduction by YY males produces all male offspring regardless if they mate with genetically normal or XY-females (Williamson and May 2002).

Petit et al. (1997) identified a number of pollutants that have estrogenic properties in salmonid fish bioassays. Since exposure of developing salmonids to hormones (Baker et al.1988; Chevassus et al. 1988; Devlin et al. 1994a) or pollutants (Jobling et al. 1998; Larsson et al. 2000; Afonso et al. 2002) can alter gonadal differentiation, and sex-reversed fish were observed in watersheds heavily impacted by pollution, hypotheses regarding Chinook salmon with altered sexual differentiation owing to exposure to

endocrine disrupting chemicals were put forward by previous studies (Nagler et al. 2001; Williamson and May 2002). However, other possibilities exist. In 2001, Nagler et al. also suggested that translocation of a region of Y-chromosome containing the OtY1 sequence to another chromosome may have occurred. Similarly, Chowden and Nagler (2004), suggested that female Chinook salmon that test positive for OtY1 are not phenotypically sex-reversed males, but are in fact genetic females, and that the observed incongruence between sexual genotype and phenotype is evidence of past genetic rearrangement involving the Y-chromosome. Water temperature fluctuations during early development have been hypothesized as another possible cause of altered sexual differentiation. However, Nagler et al. (2003) showed no significant sex ratio differences in spring-run Chinook salmon as a result of daily temperature changes that occur during the embryonic period encompassing the time when the gonad differentiates sexually.

The organization of OtY1 on the Y-chromosome increases the chance of it being involved in a genetic rearrangement. The OtY1 male sex-specific fragment is part of an 8 kb repeat sequence that occurs approximately 300 times as a head to tail tandem array comprising 2.4 Mb of the Y-chromosome (Devlin et al. 1991, 1994a, 1998). Recombination between the sex chromosomes (or the Y chromosome and an autosome) that occurs within the repetitive region could carry copies of OtY1 away from the Y-chromosome, and relative to a single-copy locus OtY1 has a greater chance of being involved in a recombinatorial event by virtue of its high copy number. The tandem arrays containing OtY1 are localized on the distal end of an acrocentric chromosome considered to be the Y-chromosome in Chinook salmon (Stein et al. 2001). If a

translocation occurs near or within this region, many copies of OtY1 as well as the GH-Ψ locus could be moved from one chromosome to another.

Salmonid sex chromosomes are in an early state of differentiation. Previous studies of Chinook salmon (Devlin et al. 2001) and other salmonids (Hunter et al. 1982, Chevassus et al. 1988) have shown that YY individuals are viable and fertile suggesting that the Y-chromosome has not degenerated to the point that it lacks vital genes present on the X-chromosome. Differentiation between the salmonid sex chromosomes is likely limited to the region immediately adjacent to the sex-determining region and the remainder of these chromosomes retains sufficient homology so that genetic exchange may still occur (May et al. 1989; Allendorf et al. 1994).

Controlled breeding experiments used to evaluate the inheritance pattern of sex-specific markers using fish with discrepant sexual genotype and phenotype have not been performed in any previous study. Original analysis of the inheritance pattern of OtY1 involved controlled crosses of Chinook salmon obtained from British Columbia populations (Devlin et al. 1991, 2001). Moreover, a survey of regional variation of Y-chromosomal DNA markers in Chinook salmon populations across the Pacific Northwest (Devlin et al. 2004) revealed a north to south cline of increasing incidence of phenotypic males and females that had an incongruent genotype at one or both markers. Chowden and Nagler (2004) stated that the potential exists that OtY1 is an inconsistent genetic marker for sex in the more southern populations of Chinook salmon. By evaluating the pattern of inheritance of the markers in controlled breeding experiments with phenotypic female Chinook salmon that have a male genotype (XY-females) one would be able to discern whether or not sex-reversed Chinook salmon are due to endocrine disrupting

chemicals in the environment or are the product of a genetic rearrangement involving the Y-chromosome. In this study we use two previously developed Y-chromosome markers, OtY1 (Devlin et al. 1991, 1994b) and the growth hormone pseudogene (Du et al. 1993), in conjunction with controlled breeding experiments to test the hypothesis of no difference from a 1:1, male to female, genotypic and phenotypic sex ratio in offspring produced between genotypically normal Chinook salmon, or between genotypically normal males and apparent sex reversed males (XY-females).

Methods

Sample collection for artificial crosses– Fin-clips for genetic analysis and gametes from fall-run Chinook salmon returning to the Merced River Fish Hatchery (MRH) were collected with the assistance of California Department of Fish and Game personnel between November and December 2003. Approximately 2 cm^2 of caudal fin tissue near the caudal peduncle was excised with scissors from each fish sampled and placed into separate, labeled coin envelopes. Between samples the scissors were mechanically cleaned and rinsed in clean running water to prevent cross contamination between the DNA of individual fish. Eggs from phenotypic females were expressed into pre-labeled plastic urine analysis cups, sealed and immediately placed on a raised platform within an ice chest. Milt from phenotypic males was expressed into labeled Zip-Loc® bags and similarly stored. Tissue samples and gametes were stored between 5-8^oC while transported back to the University of California Davis Genomic Variation Lab (GVL) for genetic analysis and use in controlled breeding experiments conducted at the Center for Aquatic Biodiversity and Aquaculture (CABA), respectively.

Genetic screening to detect apparent sex-reversed male (XY female) fish – The selection criterion for sets of gametes to be used in artificial crosses was based on the sexual genotype at the Growth Hormone pseudogene and OtY1 loci of putative parents. Genomic DNA from fin-clips was extracted using a QIAgen DNA extraction kit. Unused portions of fin-clip samples were oven dried at 32^{o}C, for 15-18 hours and then placed within the GVL sample repository. All phenotypic female and male fall-run Chinook salmon sampled were screened by polymerase chain reaction (PCR) assays using the OtY1 primers developed from Chinook salmon by Devlin et al. (1991, 1994b) and an alternate version (E. LaHood, National Marine Fisheries Service) of the Growth Hormone pseudogene (GH-Ψ) primers developed by Du et al. (1993). The alternate primer set contains the primers originally developed for GH-Ψ and includes a control primer (5'-GTT CCT CCT GAC GTT GCC GTC G-3'), which produces an 84 bp control band in Chinook salmon when used in conjunction with the forward primer. The control band alleviates the potential of obtaining a false negative signal due to chance failure of an individual PCR reaction. Both the OtY1 and GH-Ψ forward primers were 5'-end labeled with the fluorophores FAM and TET, respectively. Assays for each genetic marker were carried out separately using 20 ng of genomic DNA, 1.25 and 1.75 mM $MgCl_2$ for OtY1 and GH-Ψ, respectively, 0.2 mM each dNTP, 0.1 μM of each PCR primer, and 0.25 Units of T_{aq} DNA Polymerase, 20 mM Tris (pH 8.5) and 50 mM KCl in 10 μl volumes. Amplification of the OtY1 locus was performed in a PTC100 thermal cycler (MJ Research, San Francisco, California) under the following conditions: one denaturation cycle at 95^{o}C for 210 seconds, 35 amplification cycles of 95^{o}C for 60 s,

157 55°C for 30 s, 72°C for 60 s, and a final extension cycle of 72°C for 30 s. Amplification
158 of the GH-Ψ locus was performed under the following conditions: one denaturation cycle
159 at 95°C for 210 s, and 35 amplification cycles of 95°C for 60 s, 60°C for 60 s, 72°C for
160 120 s. DNA fragments amplified by PCR were resolved on a 5.5% acrylamide-7M Urea
161 gel and imaged by a MJ Research BaseStation (MJ Research, San Francisco, California).
162 Individual genotypes were scored using Cartographer® software as well as manually
163 verified for every individual genotyped.
164 Chinook salmon that tested positive for a 209 base pairs (bp) PCR fragment and
165 that did not produce a series of larger PCR products characteristic of the OtY1 locus in
166 females (Devlin et al. 1994) and that tested positive for a 276 bp band indicative of the
167 GH Ψ (Du et al. 1993) were scored as being positive for having the Y-chromosome
168 markers (genetic males). In the case where a fish that had ovaries that produced both a
169 robust 209 bp PCR fragment (OtY1) and the 276 bp fragment (GH-Ψ), that fish was
170 scored as a XY-female. When the larger PCR fragments characteristic of females were
171 present and the 209 bp PCR fragment was not present and the 276 bp band indicative of
172 the GH pseudogene was also absent, the fish was scored as being negative for having the
173 Y-chromosome markers (genetic female).
174
175 *Breeding experiments* - The eggs from each single phenotypic female fish selected were
176 split into roughly two equal portions and placed into separate styro-foam containers.
177 Aliquots of eggs were separately fertilized with milt from separate, single, genetically
178 normal males. In this manner, each family of fish had only two parents. Artificial
179 crosses were performed in the following manner. Approximately 0.2 ml of milt was

transferred to the eggs using a sterile pipette. Eggs were gently swirled with a clean, dry, latex gloved finger to coat them evenly in milt. Enough 12^{o}C water was added to just cover the eggs and activate the sperm. The gametes were gently swirled for a few seconds to insure adequate mixing and were allowed to incubate 30-45 s. More 12^{o}C water was added to allow for swelling of fertilized eggs. Eggs were incubated for approximately 15 minutes to allow them to begin water hardening. During this incubation the water was changed once to keep the eggs cold and oxygenated. An equal volume of a 1:100 dilution of Argentyne® iodofore disinfectant (Scubla Aquaculture, Udine Italy) in 12^{o}C water was added to the eggs for 10 min. as a prophylaxis against possible contamination with infectious viruses (i.e.- infectious hematapoietic necrosis virus). Fluid was decanted from the eggs and eggs were rinsed twice with 12^{o}C water, and each 'family' was transferred to separate, labeled hatch-out (Heath) trays supplied with 12^{o}C running water. No more than 30 hours elapsed between the harvest of eggs at the Merced River Fish Hatchery and their fertilization at CABA.

Dead eggs, developing embryos, and alevins were removed on a daily basis to prevent development of bacterial or fungal growth in the incubation trays. Specimens were stored in 100% ethanol at 4^{o}C until processed for genotyping. Although the gonad morphology of these specimens could not be ascertained due to the fact that the gonads have not yet developed to a point where they can be visually differentiated between the sexes, these specimens were genotyped using both the OtY1 and GH-Ψ Y-chromosome markers. Hatchlings from individual families were incubated at 12^{o}C for approximately 45 days (just before swim up stage) in the Heath trays before being transferred to separate, larger rearing tanks. The tanks were equipped with screens to prevent

inadvertent cross contamination of families by escaped individuals from adjacent tanks. During the rearing period, juvenile fish were fed a commercially available diet twice daily. Any dead parr were removed and stored in 100% ethanol at 4^{o}C until processed for genotyping. Juvenile fish were raised to a fork length of approximately 160 mm (approximately 120-140 days post-fertilization) before they were euthanized for dissection. Juveniles were euthanized by anesthetic overdose via immersion in 12^{o}C water containing 500 mg/l tricaine methanesulphonate (MS-222, Argent Laboratories, Redmond, WA). Euthanized specimens were stored on ice until they could be dissected the same day.

Observation of gonad gross morphology in sampled juvenile Chinook salmon -

The gonads of juvenile fish were visualized with a dissection microscope (60X magnification). Two incisions were made to allow access into the body cavity. The first ventral incision extended from the gill isthmus to the vent and a second lateral incision starting immediately posterior of the dorsal fin into the dorsal musculature were made. As was noted by Jensen & Hyde (1971), great care must be exercised to not perforate the swim bladder since detection of the gonads is very difficult once the swim bladder has been deflated. In juvenile phenotypic males, the immature testes appear as two long, flattened, smooth, approximately 1 mm wide, translucent white, tubular organs that run along the ventral surface of and are closely associated with the swim bladder. The immature ovaries of juvenile phenotypic females appear as two, long, roughly triangular, opaque yellowish-white, tubular organs that are granular in appearance and texture. The ovaries are approximately 2-3 mm wide at the apical end and quickly taper to ribbon-like

structures that run along the ventral surface of and are closely associated with the swim bladder. The granular appearance of the immature ovaries is due to the presence of developing ova. Gross morphology of both gonads was verified by necropsy for all individuals except in those families where all individuals had died before gonad development had progressed to a point at which phenotypic sex could be ascertained.

Statistical analysis - Genotyping data was examined for consistency of Y-chromosome marker scores within individuals and was statistically tested to evaluate the offspring genotypic sex ratio in each family. The offspring phenotypic sex ratio of each family, based on internal observation of gonad gross morphology, was evaluated in the same manner. The consistency of sex marker scores was evaluated by merely observing whether or not the genetic markers corroborated one another in each individual tested. A Chi-square Goodness of Fit analysis, utilizing a Yates (1934) correction for continuity to prevent inflating the probability of committing a Type I error, was used to determine if the observed genotypic and phenotypic sex ratios from individual families deviated significantly (α=0.05) from what is expected under a null hypothesis. For control crosses between genotypically normal Chinook, or experimental crosses between genotypically normal males and apparent sex reversed males (XY-females), the null hypothesis of no difference between a one male to one female sex ratio in the sampled progeny was tested. The Chi-square Goodness of Fit analyses of offspring sexual genotype were performed on families where the phenotypic sex of offspring could be ascertained as well as those families whose offspring had died prior to developing to a point where gonad gross morphology could be evaluated with certainty.

Results

In none of the 2384 adults and progeny evaluated did the GH-Ψ and OtY1 Y-chromosome specific markers contradict one another. The tight linkage observed between these two markers in Chinook salmon is similar to that observed by Devlin et al. (2001). A total of 156 adult fish (135 and 21 phenotypic females and males, respectively) collected from the MRH were genotyped. Genetic analysis revealed 8 out of the 135 (~6%) phenotypic females examined had a male genotype according to GH-Ψ and OtY1. The remaining 127 phenotypic females screened were genetic females, and all 21 phenotypic male fish screened were genetic males according to both genetic markers.

A total of 622 dead embryos, alevins, and parr (< 40 mm fork length) whose phenotypic sex were not evaluated since gonadal development had not proceeded to a point that it could be ascertained were genotyped using both GH-Ψ and OtY1. These mortalities had occurred in all families prior to commencing dissections of the offspring that had developed for 120-140 days. The two genetic markers corroborated one another in all individuals tested.

Families 87xB and 87xD had offspring genetic sex ratios that deviated significantly from a 1:1, male to female, ratio (Table 1). These families, originating from XY-female #87, suffered very high early mortality within 72 hours of fertilization. All of the individuals analyzed from these two families were either dead embryos or alevins that had developed for a sufficient period of time to permit the harvest of an adequate amount of tissue for genetic analysis of sexual genotype. The 16 offspring analyzed from family 93xB were either dead embryos or alevins as well. High mortality prior to hatch was also observed for families from genetically normal females collected the same day as XY-

female #87. Mike Kozart (Mgr., Merced River Fish Hatchery, CDFG, personal communication) observed high mortality of eggs at the Merced River Fish Hatchery that were collected on the same date as female #87. It is possible that the elevated mortality observed in these families was due to the spike in water temperature that had occurred just prior to and during the collection date for these fish (Mike Kozart, personal communication). The water source of the Merced R. Fish Hatchery is the Merced River.

A total of 1606 individuals from 14 out of 17 artificial crosses successfully reared for 120-140 days were genotyped and dissected to verify gonad gross morphology. No statistically significant deviations from 1:1, male to female, phenotypic or genotypic sex ratios were observed in 6 out of 7 families (Table 1) or in all families combined (X^2 = 0.03, df 1, p=0.85) from genetically normal, phenotypic female parents. All phenotypic male offspring from these families were positive for both Y-chromosome markers and all phenotypic female offspring were negative for both markers. Family 105xB, the sole exception, had a significantly higher ($p<0.05$) number of phenotypic female offspring than would be expected by chance alone (Table 1). Male and female offspring of this family did not suffer mortality differentially and the reason for the significantly different sex ratio observed is unknown. Here too sexual genotype of both markers was in concordance with sexual phenotype in all offspring.

Seven families produced by XY-females had enough offspring survive so that both sexual genotype and phenotype could be ascertained. Observed offspring phenotypic sex ratios of the seven families combined differed significantly (X^2 = 6.61, df 1, p=0.01) from a 1:1, male to female, sex ratio. The significant difference from a 1:1 phenotypic sex ratio in offspring produced by XY-females was due to two families, 93xA

and 118xC. Both had significantly higher ($p<0.05$) numbers of phenotypic male offspring and each exhibited some to the most highly skewed genotypic sex ratios (Table 1). Neither of these two families suffered early mortality differentially between the sexes. No significant deviation from a 1:1, male to female, offspring phenotypic sex ratio was observed when the five remaining families produced by XY-females were combined ($X^2 = 2.26$, df 1, $p=0.13$), or assessed individually (Table 1).

Unlike the families produced by genetically normal phenotypic females discrepancies between sexual genotype and phenotype were observed in the offspring of XY-females. Statistically significant deviations ($p<0.05$ or 0.001) from a 1:1, male to female, genotypic sex ratio (Table 1) were observed in 9 out of 10 families produced by XY-females. Since the sample size of 93xB was small (N=16), the power to detect a significant deviation from the expected 1:1, male to female, genotypic sex ratio was low. Hence, family 93xB lacked statistical significance ($0.10>p>0.05$) despite having a 3:1, male to female genotypic sex ratio (Table 1). The observed offspring genotypic sex ratio of the combined seven XYF families (non-asterisked in Table 1) was significantly different ($X^2 = 327.94$, df 1, $p<0.0001$) from 1:1, males to females, and it was significantly different from the offspring genotypic sex ratio observed in the combined families produced by genetically normal phenotypic females which did not differ significantly ($X^2 = 0.03$, df 1, $p=0.88$) from 1:1, males to females. All phenotypic male offspring produced by apparent XY-females were genotypically male at both Y-chromosome markers. Roughly one half of the phenotypic female offspring produced by apparent XY-female parents were genotypically male at both markers. The remaining phenotypic female offspring were genotypically female at both markers. In short, the

excess of male genotypes at both GH-Ψ and OtY1 in offspring produced by apparent XY-female fish is due to roughly half of the phenotypic female offspring of these crosses being scored as males.

The genotypic sex ratios of offspring produced by 'apparent' XY-females ranged from 2.55:1 to 4.93:1 (average 3.7:1), males to females. The observed genotypic sex ratio of offspring produced by XY-female families did not significantly differ from 3:1, males to females (Table 1). However, when the XY-female families (non-asterisked in Table 1) were combined, a significantly higher number of male genotypes ($X^2 = 9.06$, df 1, $p=0.002$) were observed than would be expected by chance alone from a 3:1, male to female, sex ratio. The detected deviation from a 3:1 genotypic sex ratio for the combined XY-female families is due in part to increased power via an increase in sample size.

Discussion

Observed incongruence between sexual genotype and phenotype in half of the phenotypic female progeny of apparent XY-females strongly suggests that a genetic rearrangement or mutation rather than altered sexual development due to endocrine disruption is responsible for the XY-female fall-run Chinook salmon in the Central Valley. Accordingly, neither of the two Y-chromosome markers used in this study are 100% diagnostic of female sex in Central Valley fall-run Chinook salmon. The observed 1:1, male to female, offspring phenotypic sex ratio was not the expected 3:1 ratio as would be obtained from the cross between a true sex-reversed male (XY-female) and a normal (XY) male (Figure 1C). If, as was hypothesized by Williamson and May (2002), apparent XY-females were the result of altered sexual differentiation, owing to exposure

342 to environmental EDCs, one would not expect to observe incongruent sexual genotype
343 and phenotype within individual progeny of these fish. If the developmental pathway(s)
344 controlling sexual phenotype were altered by exposure to EDCs at a critical period of
345 development such changes may occur without necessarily also eliciting a heritable
346 change in the germ cells of exposed individuals. Hence, previous reports (Nagler et al.
347 2001; Williamson and May 2002) regarding altered sexual differentiation in Chinook
348 salmon due to endocrine disruption were incorrectly interpreted.
349
350 Several genetic rearrangements are possible that would explain the observed
351 pattern of inheritance. First, there may have been a recombination event (or
352 translocation) between the Y- and X-chromosome or an autosome that carried along
353 copies of both the OtY1 and GH-Ψ loci. Phenotypic female fish carrying an autosome or
354 X-chromosome to which a portion of the Y-chromosome bearing the markers has
355 translocated, or carrying a Y-chromosome bearing a mutation or deletion that
356 functionally inactivates the sex-determining region (Figures 1A and 1B, respectively)
357 would, when mated to a normal male, produce offspring with a 3:1, male to female,
358 genotypic sex ratio while maintaining a 1:1 phenotypic sex ratio. In either scenario half
359 of the phenotypic female offspring would bear a male genotype since they have a 50%
360 chance of inheriting the recombinant chromosome or translocation from the phenotypic
361 female parent. Inactivation of the sex-determining region on the Y-chromosome can
362 occur due to a frame shift and/or nonsense mutation as a result of an insertion or deletion
363 mutation. Matsuda et al. (2002) described a nonsense mutation in medaka fish (*Oryzias*
364 *laptipes*). The Awara mutation is a single nucleotide insertion in exon 3 of *DMY* that

365 causes a frame shift and subsequent truncation of DMY. All offspring that inherited the
366 Awara mutant allele of *DMY* were female (Matsuda et al. 2002). Alternatively, decreased
367 expression of a gene that plays a role in differentiation of the bipotential gonad may be
368 responsible. The Shirone mutation in Medaka (Matsuda et al. 2002) leads to a very low
369 expression of *DMY* resulting in a high proportion of XY females in fish that carry the
370 mutation.

371 The genetic rearrangement or mutation responsible for producing 'apparent' XY-
372 female fall-run-Chinook salmon in the Central Valley has likely arisen independently of
373 any such genetic changes that have occurred in other more northern populations of
374 Chinook. In this study all phenotypic males and apparent XY-females had a clearly
375 defined male genotype according to both OtY1 and GH-Ψ. All phenotypic females
376 categorized as normal were negative for both male markers. In contrast, Devlin et al.
377 (2004) observed low numbers of phenotypic females and males that were positive for
378 only either OtY1, or GH-Ψ, and phenotypic males negative and phenotypic females
379 positive for both markers. Devlin et al. (2004) described weakly amplifying allelic
380 variants for both loci in males and females, and they suggest recombination, deletion, and
381 PCR priming site sequence variation (in the case of allelic variation) of OtY1 copies and
382 the GH-Ψ locus as possible explanations for the observed variation. The far lesser degree
383 of sex marker variation observed in California Chinook salmon (Williamson and May
384 2002) and the fact that GH-Ψ is a single copy marker (Du et al. 1993) suggests that a
385 single genetic change different from those in more northern populations has occurred. If
386 a translocation of markers from the Y- to X-chromosome occurred, it will have likely
387 happened only once since translocations are rare events.

Only when all XY-female families are combined, is there a higher than expected number of male genotypes (relative to a 3:1, male to female, sex ratio). An explanation may be that chromosomes carrying the genetic alteration may have a slight fitness advantage relative over their homologous, wildtype counterparts. The delay of the second meiotic division in females coupled with partitioning of chromatids to the polar bodies may play a role in the process. If the genetic alteration does confer a chromosomal fitness advantage (or at least a relatively lower probability of being shunted to a polar body) then its frequency within a population may likely increase over subsequent generations. This may explain, in part, how an alleged single mutation could spread throughout the Central Valley.

Current data from the breeding experiments do not indicate which of the alternate models (Figures 1A and B) is more likely. Fluorescent In Situ Hybridization (FISH) assays performed with OtY1 and GH-Ψ (Devlin et al. 1991, 1994b; Du et al. 1993, respectively) can be used to probe lymphocyte chromosome spreads obtained from the offspring of phenotypic females positive for both Y-chromosome markers and genetically normal, phenotypic females. Comparison of chromosome staining patterns obtained from the offspring of normal and 'apparent' XY-females may provide a way to differentiate which chromosomal mechanism is responsible for producing apparent XY-female fall-run Chinook salmon in the Central Valley. This assumes, however, that the FISH assay can provide sufficient resolution to differentiate between chromosome staining patterns produced by a Y- to X-chromosome/autosome translocation and an intact, normal Y-chromosome.

The FISH methodology would not differentiate between a wild-type Y-chromosome, one lacking a functional sex-determining locus due to a small indel mutation, or a chromosome translocation, particularly if the translocated region is large. In this case, other molecular genetic techniques may provide a means to differentiate between the proposed alternate models. DNA strand differences may be observed by performing chromosome walks on OtY1 and GH-Ψ positive clones of bacterial artificial chromosome libraries created from a phenotypic female offspring positive for the markers and a phenotypic male offspring from a control cross. Alternatively, suppressive subtractive hybridization (SSH) performed on genomic DNA isolated from phenotypic female offspring that do and do not carry the rearrangement/mutation can be used to create a DNA library enriched for recombinant sequences (in the case of a recombinant or translocated chromosome), or the non-wild type Y-chromosome (in the case of a mutation on the Y-chromosome). Sequence data obtained from SSH enriched library clones positive for OtY1 and GH-Ψ may then be compared to similarly positive clones from a library created from a phenotypic male offspring that does not carry the rearrangement/mutation. DNA sequences that differ between subtracted and non-subtracted library clones may be evaluated by testing their segregation pattern in offspring of normal and 'apparent' XY-females. Resolution of the genetic mechanism responsible for the two types of phenotypic females should help us to understand sex determination in salmonid fishes.

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Table 1. - Observed genotypic and phenotypic sex ratios of progeny in individual families produced from breeding experiments. Phenotypic female parents that were genotypically female, or male at both Y-chromosome markers are designated as normal or XY-female (XYF), respectively. P-values are designated as a: $p<0.05$, or b: $p<0.001$. Gross morphology of gonads was verified by necropsy for all individuals except in those families where all individuals had died before gonad development had proceeded to a point at which phenotypic sex could be ascertained (*). Chi-squared test results for 1:1 and 3:1, male to female, genotypic sex ratios are shown in columns 7 and 8, respectively. Counts of phenotypic males (PM) with male genotype (GM), phenotypic females (PF) with male genotype, and phenotypic females with female genotype (GF) are shown in the last three columns.

Family ID	Phenotypic Female Parent	N	Observed Phenotypic Ratio (M:F)	Phenotypic Chi-square Value	Observed Genotypic Ratio (M:F)	Genotypic Chi-square Value (1M:1F)	Genotypic Chi-square Value (3M:1F)	Observed Counts PM GM	PF GM	PF GF
84 x B	normal	92	1.14 : 1	0.27	1.14 : 1	0.27	--	49	0	43
85 x B	normal	82	1.41 : 1	0.99	1.41 : 1	0.99	--	48	0	34
85 x D	normal	69	1.09 : 1	0.38	1.09 : 1	0.38	--	36	0	33
105 x A	normal	96	1.18 : 1	0.51	1.18 : 1	0.51	--	52	0	44
105 x B	normal	96	0.62 : 1	4.59[a]	0.62 : 1	4.59[a]	--	37	0	59
112 x D	normal	96	0.83 : 1	0.51	0.83 : 1	0.51	--	44	0	52
128 x D	normal	90	0.83 : 1	0.27	0.83 : 1	0.27	--	41	0	49
87 x B*	XYF	40	--	--	3.44 : 1	11.02[b]	0.01	--	--	--
87 x D*	XYF	32	--	--	2.55 : 1	5.28[a]	0.04	--	--	--
93 x A	XYF	106	1.35 : 1	5.63[a]	4.30 : 1	39.86[b]	1.81	61	25	20
93 x B*	XYF	16	--	--	3.00 : 1	3.06	0.08	--	--	--
118 x C	XYF	83	1.68 : 1	4.89[a]	4.93 : 1	35.57[b]	2.51	52	17	14
118 x D	XYF	144	1.18 : 1	0.45	3.50 : 1	43.34[b]	1.25	78	34	32
126 x C	XYF	154	1.26 : 1	0.94	4.31 : 1	58.60[b]	2.80	86	39	29
126 x D	XYF	142	1.09 : 1	0.17	4.26 : 1	53.30[b]	2.40	74	41	27
130 x A	XYF	152	0.91 : 1	0.32	2.62 : 1	29.53[b]	0.43	72	38	42
130 x D	XYF	116	1.23 : 1	1.04	4.04 : 1	41.04[b]	1.39	64	29	23
	Grand total:	**1606**								

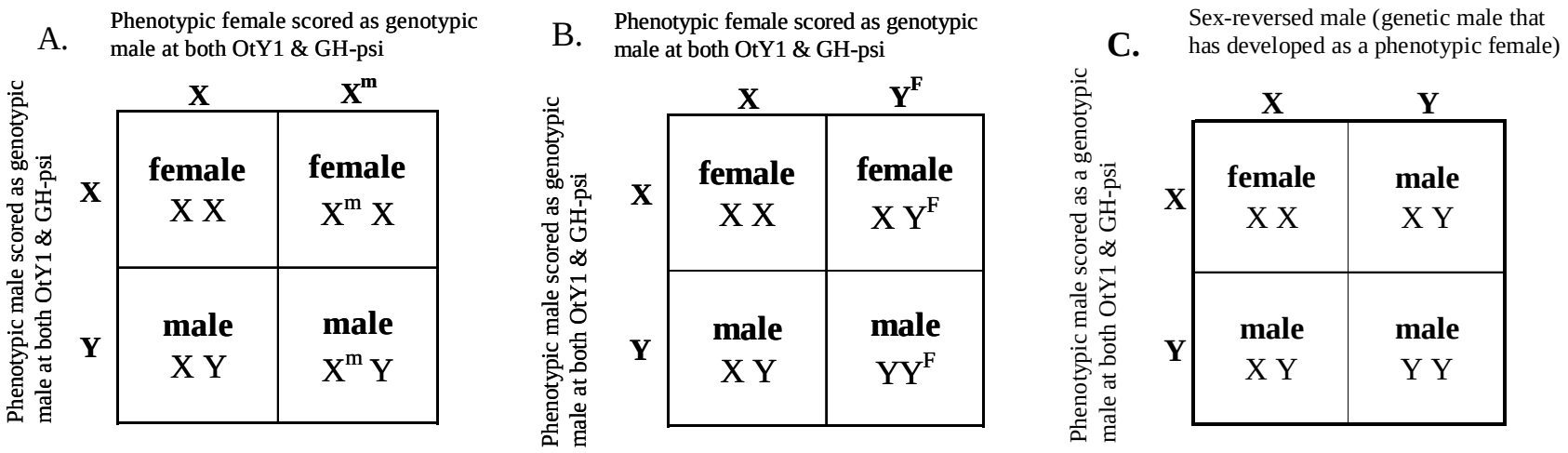

Figure 1. - Alternate models to explain incongruence of genotypic and phenotypic sex in half of the phenotypic female offspring produced by apparent XY-female fall-run Chinook salmon. Punnet squares depicting the possible offspring produced between a normal male and a phenotypic female parent carrying an X-chromosome with a translocated portion of the Y-chromosome designated by X^m (A), or a dysfunctional Y-chromosome designated by Y^F (B) are shown. Both models predict the phenotypic and genotypic sex ratios (1:1 and 3:1, male to female, respectively) observed in offspring from XY-females used in breeding experiments. A mating between a true sex-reversed male and a normal male (C) would produce a 3:1, male to female, genotypic and phenotypic sex ratios in the offspring. Wild type X- & Y-chromosomes designated by X and Y, respectively. Sexual phenotype of offspring is in bold.